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Patentanmeldung Nr. Patent application No. Demande de brevet n°

98106302.7

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**Blatt 2 der Bescheinigung
Sheet 2 of the certificate
Page 2 de l'attestation**

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NEW COMPOUNDS FOR DNA-TRANSFECTION

The present invention is concerned with novel compounds especially useful for non-viral DNA transfection.

Non-viral systems have been developed to carry DNA into cells, e.g., the
5 transfection technique based on a cationic lipid, the dioleoyloxypropyl trimethyl-
ammonium (Felgner et al., Proc. Natl., Acad. Sci. USA, 1987, 84, 7413-7417,) commercialized as Lipofectin™. Since the discovery of this transfection technique, many more cationic lipids have been synthesized and some are commercially available as transfecting reagent for laboratory use: DOGS (Transfectam™), DOSPA
10 (Lipofectamine™), DOTAP (DOTAP™).

Transfection of cells with oligonucleotides such as DNA can be used, for example to express in a host cell or organism, a protein which is not normally expressed by that cell or organism. For example, a self replicating DNA molecule called a plasmid may be introduced into a cell not normally containing that plasmid in order to express a marker
15 gene product in that cell, or to express a protein of interest such as a recombinant protein which is later harvested from such cells. (See Sambrook, et al., Molecular Cloning: A Laboratory Manual, 2nd ed. (Cold Spring Harbor, 1989), ch. 1.) The transfection of oligonucleotides into cells can also be used therapeutically. For example, antisense oligonucleotides, once in the cell or cell nucleus, bind to target single-stranded nucleic acid
20 molecules by Watson-Crick base pairing or to double stranded nucleic acids by Hoogsteen base pairing, and in doing so disrupt the function of the target by one of several mechanisms: by preventing the binding of factors required for normal transcription, splicing, or translation; by triggering the enzymatic degradation of mRNA by RNase H, or by destroying the target via reactive groups attached directly to the antisense
25 oligonucleotide. (See Zamecnic et al., Proc. Natl. Acad. Sci.-USA, 1978, 75, 280-284). Gene therapy or DNA based vaccination are other therapeutic applications.

Proteins and other anionic macromolecules are transfected into cells for therapeutic and screening purposes. For example, immunization is enhanced by introducing an

immunogenic protein into a cell, so that it is more efficiently processed and presented on the surface of the cells, thereby enhancing the immunogenic response. Negatively charged anionic macromolecules which act inside a cell are transported past the cell membrane into the cytoplasm where they exert their effect. Factors which enhance or hinder transcription of DNA can be used in a screening test to verify the transcription of a gene of interest. These transcription assays are very well known for use in screening compounds to determine their effect against a particular macromolecule, for example a cell receptor.

Cell-lytic antibacterial peptides that act by perturbing the barrier function of membranes are reviewed in Saberwal, et al., *Biochim. Biophys. Acta*, 1994, 1197, 109-131. Certain fatty acid-bearing basic peptides having antibacterial activity are disclosed in Vogler, et al., *Helv. Chim. Acta*, 1964, 47, 526-543. Poly(lysine-serine) random polymers for use as carriers to transport oligonucleotides into a cell is disclosed in European Patent Publication No. EP-A-727 223.

European Patent Publication No. EP-A-784 984 and Legendre et al. (*Bioconjugate Chem.*, 1997, 8, 57-63) describe conjugates of a lipid and a basic, membrane disturbing peptide which bind polynucleotides and anionic macromolecules and can be used for transfection of cells. The peptide portion of the conjugate consists of a natural amino acids linked by a natural amide binding.

Nevertheless, despite an important progress in the formulation of non-viral gene delivery systems, there remains a need for more efficient techniques, since the transfection efficiency of synthetic systems is usually below that of viral vectors. Furthermore, still many problems arise in vivo and the poor stability of the non-viral systems in biological fluids does not allow high and reproducible levels of transfection in vivo.

Many known compounds used for transfection experiments are to a certain extent toxic for eucaryotic cells. Often problems occur in scaling up where the conditioned medium reduces the effect of known transfecting agents.

The present invention is directed to novel compounds which avoid disadvantages associated with known transfection agents.

In one aspect, the invention is concerned with novel compounds which are conjugates of lipids and basic, membrane disturbing peptides, characterized in that the peptides comprise a reversed amide backbone.

The term "conjugates" means compounds consisting of a lipid chemically bound to the peptide, e.g., via a disulfide bond formed between a sulfur atom present in or attached

to the lipid and a sulfur atom present in or attached to the peptide; or an amide bond formed between the carboxyl group present in or attached to the lipid and an amino group of the peptide.

5 The term "lipid" as used herein comprises straight-chain, branched-chain, saturated or unsaturated aliphatic carboxylic acids and phospholipids. Examples of aliphatic carboxylic acids are lauric acid, palmitic acid, stearic acid, oleic acid and $(\text{CH}_3(\text{CH}_2)_n)_2\text{CHCOOH}$, where n is an integer from 3 to 19. Examples of phospholipids are phosphatidylethanolamines such as dioleoylphosphatidylethanolamine.

10 The term "basic peptides" denotes peptides containing at least one basic amino acid. Examples of such basic amino acids are natural and unnatural diamino-monocarboxylic acids, such as alpha, beta-diaminopropionic acid, alpha, gamma-diaminobutyric acid, lysine, arginine, ornithine and p-aminophenylalanine, etc.

15 The term "membrane disturbing peptides" denotes cell-lytic or antibacterial peptides which perturb the barrier function of membranes (G. Saberwal and R. Nagaraj, BBA, 1994, 1197, 109-131). Examples of basic, cell-lytic peptides are melittin, hemolysin, mastoparan, bombolitin, crabrolin and derivatives thereof. Examples of basic antibacterial peptides are cecropins, magainins, gramicidin S and tyrocidine and derivatives thereof.

20 The term "derivatives" refers to peptides wherein one or more amino acid residues are missing or have been replaced by another amino acid residue without substantially changing the biological activity of the original peptide concerned, i.e. allow transfection of a macromolecule, preferably a polynucleotide, into a cell. The term "derivatives" also refers to peptides wherein the terminal carboxyl group is esterified, particularly to form lower alkyl esters such as the methyl and ethyl ester; or converted into an amide, lower alkyl amide or di-lower alkyl amide or hydrazide. The term "derivatives" also relates to
25 peptides wherein the NH_2 -group of the N-terminus may be acylated to form an amide or a lower alkyl amide. In particular, the NH_2 -group is acetylated. The term "lower" denotes groups containing from 1-6 carbon atoms.

30 The term "reversed amide backbone" refers to retro-peptides which characteristically have the same side chain sequence as the parent peptide, $-\text{CO}-\text{NH}-$, but the amide bond direction is reversed: $-\text{NH}-\text{CO}-$.

Throughout this application the following standard abbreviations are used to refer to amino acids:

Alanine

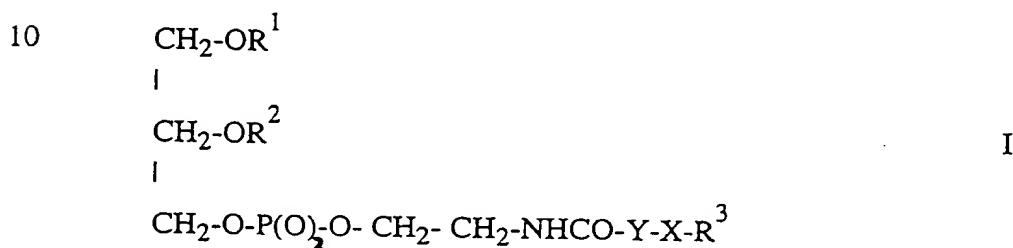
Ala

A

	Glutamate	Glu	E
	Glutamine	Gln	Q
	Aspartate	Asp	D
	Asparagine	Asn	N
5	Leucine	Leu	L
	Glycine	Gly	G
	Lysine	Lys	K
	Serine	Ser	S
	Valine	Val	V
10	Arginine	Arg	R
	Threonine	Thr	T
	Proline	Pro	P
	Isoleucine	Ile	I
	Methionine	Met	M
15	Phenylalanine	Phe	F
	Tyrosine	Tyr	Y
	Cysteine	Cys	C
	Tryptophan	Trp	W
	Histidine	His	H
20	Ornithine	Orn	O
	D-Alanine	D-Ala	a
	D-Glutamate	D-Glu	e
	D-Glutamine	D-Gln	q
25	D-Aspartate	D-Asp	d
	D-Asparagine	D-Asn	n
	D-Leucine	D-Leu	l
	D-Lysine	D-Lys	k
	D-Serine	D-Ser	s
30	D-Valine	D-Val	v
	D-Arginine	D-Arg	r
	D-Threonine	D-Thr	t
	D-Proline	D-Pro	p
	D-Isoleucine	D-Ile	i
35	D-Methionine	D-Met	m

D-Phenylalanine	D-Phe	f
D-Tyrosine	D-Tyr	y
D-Cysteine	D-Cys	c
D-Tryptophan	D-Trp	w
5 D-Histidine	D-His	h
D-Ornithine	D-Orn	o

In a preferred embodiment, the novel compounds of the present invention are compounds of formula



wherein R^1 and R^2 are a hydrocarbonyl moiety of a straight-chain or branched-chain, saturated or unsaturated aliphatic carboxylic acid or a phospholipid moiety, R^3 is a basic, membrane disturbing peptide with a reversed amide backbone, Y is C_{2-10} alkylene, X is -C(O)-NH- or -S-S-, and salts thereof.

20 Particularly preferred compounds are those wherein R^1 and R^2 independently are an acyl moiety of a C_{12-20} carboxylic acid. The term " C_{12-20} " denotes a number of carbon atoms of from 12 to 20. The acyl moieties R^1 and R^2 can be a straight-chain or branched-chain, saturated or unsaturated moiety. Examples of such moieties are lauroyl, palmitoyl, stearoyl and oleoyl. In a preferred aspect, R^1 and R^2 are oleoyl. Y is preferably ethylene, 25 propylene or decamethylene. X is preferably -S-S-.

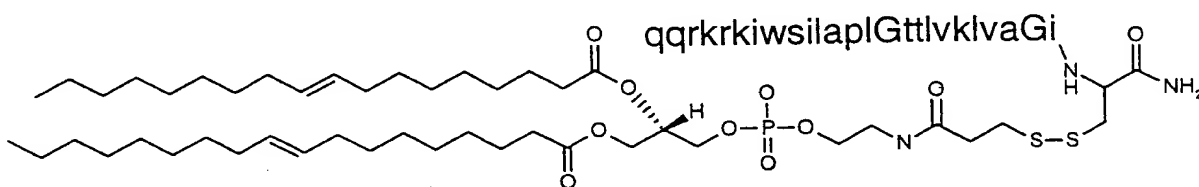
R^3 is a basic, membrane disturbing peptide with a reversed amide backbone. For example, R^3 is Gln-Gln-Arg-Lys-Arg-Lys-Ile-Trp-Ser-Ile-Leu-Ala-Pro-Leu-Gly-Thr-Thr-Leu-Val-Lys-Leu-Val-Ala-Gly-Ile-NH-CH[CONH₂]-CH₂-. The peptide has a reversed amide backbone. Preferably, R^3 comprises at least 50%, more preferably 65%, and even 30 more preferably 80% of D-amino acids or derivatives thereof. In the most preferred embodiment all amino acids are D-amino acids. The term "D-amino acid" refers to naturally as well as non-naturally D- α -amino carbonic acids or derivatives thereof. In addition R^3 also comprises membrane-disturbing peptide sequences such as magainin, cecropin, defensins, etc., or chimeras of such e.g., cecropin-melittin (Hancock & Lehrer

TIBTECH, 1998, vol. 16, 82-88) synthesized as the retro-inverso peptides and derivatized to allow conjugation to lipids, analogous to the methods as described below, especially analogous in fashion to Example 1.

- 5 In a further preferred embodiment, R³ is D-Gln-D-Gln-D-Arg-D-Lys-D-Arg-D-Lys-D-Ile-D-Trp-D-Ser-D-Ile-D-Leu-D-Ala-D-Pro-D-Leu-Gly-D-Thr-D-Thr-D-Leu-D-Val-D-Lys-D-Leu-D-Val-D-Ala-Gly-D-Ile-NH-[CONH₂]-CH-(CH₂)₂-.

The most preferred compound is

10



IV

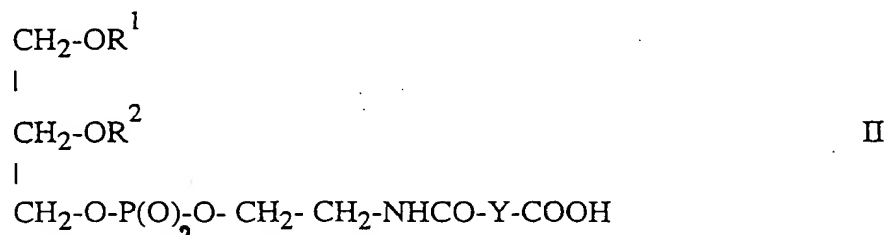
- A further embodiment of the present invention refers to the peptide portion of R³, especially to the intermediate peptide Gln-Gln-Arg-Lys-Arg-Lys-Ile-Trp-Ser-Ile-Leu-Ala-Pro-Leu-Gly-Thr-Thr-Leu-Val-Lys-Leu-Val-Ala-Gly-Ile-Cys-NH₂ or derivatives and/or salts and/or solvates thereof having a reversed amide backbone and consisting of at least 50%, more preferably 65%, and even more preferably 80% of D-amino acids or derivatives thereof. In the most preferred embodiment all amino acids are D-amino acids.
- 15 The term "D-amino acid" refers to naturally as well as non-naturally D-α-amino carbonic acids or derivatives thereof.
- 20

- In a preferred embodiment the peptide is D-Gln-D-Gln-D-Arg-D-Lys-D-Arg-D-Lys-D-Ile-D-Trp-D-Ser-D-Ile-D-Leu-D-Ala-D-Pro-D-Leu-Gly-D-Thr-D-Thr-D-Leu-D-Val-D-Lys-D-Leu-D-Val-D-Ala-Gly-D-Ile-D-Cys-NH₂.
- 25

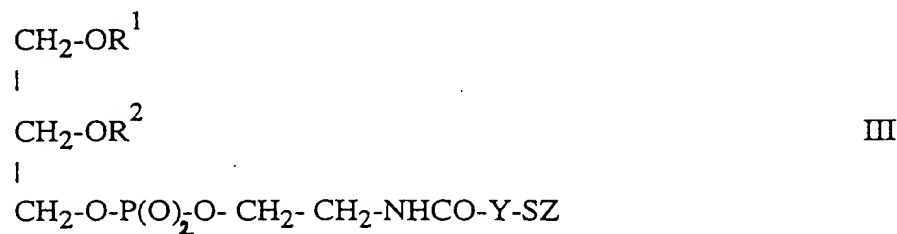
- In another aspect, this invention relates to a process for preparing the novel compounds defined above, i.e., conjugates of lipids and basic, membrane disturbing peptides wherein the peptides comprise a reversed amide backbone, and compositions comprising at least one such compound, a polynucleotide or any other anionic macromolecule, and, optionally, a helper lipid and/or a short chain phospholipid, and/or a cationic lipid or another known transfection competent molecule other than a conjugate of this invention (i.e. a compound of formula I or II). In still another aspect, this invention relates to compositions comprising conjugates of lipids and basic, membrane disturbing peptides and a helper lipid and/or a short chain phospholipid, and/or a cationic lipid or
- 30

another known transfection competent molecule other than a conjugate of this invention, e.g. a compound of formula I. The invention further relates to the use of the novel compounds as a carrier for transfecting a cell with a polynucleotide or any other anionic macromolecule.

5 The compounds provided by this invention can be prepared by reacting a peptide of the formula R^3NH_2 with a lipid of the formula



10 or a peptide of the formula R^3SH with a lipid of the formula



15 20 wherein R^1 , R^2 , R^3 and Y are as defined above and Z is a leaving group such as 2-pyridinethio. These reactions can be carried out in a manner known per se.

Compounds of the formula R^3NH_2 wherein the peptide part comprises a reversed amide backbone may be prepared by methods known in the art. The corresponding
25 methods have been described for example for the preparation of synthetic melittin, its enantio, retro, and retroenantio isomers and derivatives thereof (Juvvadi et al. (1996) J. Am. Chem. Soc. 118, 8989-8997).

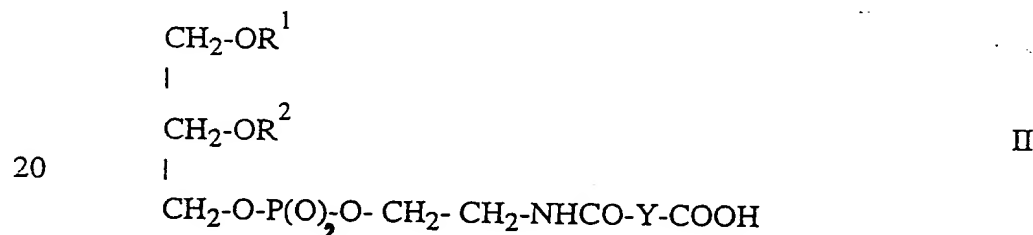
The peptides may be prepared by the solid-phase synthesis technique. In this technique, synthesis is occurring while the peptide is attached to a polymeric support,
30 therefore allowing for the separation of product from byproduct by washing steps. The completion of the acylation reaction is ensured by using large excesses of soluble reagents. Synthesis involves the covalent anchorage of the first amino acid in the sequence to the solid support followed by the deprotection of the protected amino function for the subsequent coupling to the incoming amino acid derivative. After n cycles of deprotection

and coupling, the peptide is released from the solid phase by a chemical cleavage reaction. In a preferred embodiment, D-amino acids are used for the coupling steps.

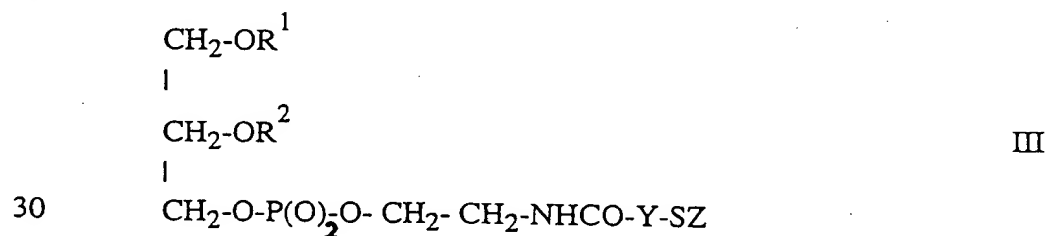
Thus, the coupling of peptide of the formula R^3NH_2 with a lipid of the formula II can be accomplished by reacting the compounds wherein amino group other than the amino group to be reacted are protected in a suitable solvent in the presence of a condensation agent such as dicyclohexylcarbodiimide in analogy to methods known for producing peptide bonds.

The reaction of a peptide of the formula R^3SH with a compound of formula R-SZ can be carried out in an appropriate solvent or solvent mixture which solubilizes both reactants. The compound of formula R-SZ can be dissolved in an organic solvent, e.g., in chloroform. The peptide R^3SH is suitably dissolved in aqueous buffer solution, such as phosphate buffer, that contains an appropriate amount of an water-miscible organic solvent such as acetonitrile to accomplish the formation of a single phase reaction system.

Compounds of the formula II and III are known or can be prepared by known methods, e.g. as described in *Biochim. Biophys. Acta*, 1986 862, 435-439. For example, compounds of the formula



wherein R^1 and R^2 are oleoyl, and Y is ethylene, propylene or decamethylene and the compound of the formula



wherein R^1 and R^2 are oleoyl, Y is ethylene and Z is 2-pyridinethio are commercially available as N-Succinyl-PE, N-Glutaryl-PE, N-Dodecanyl-PE and N-PDP-PE from Avanti Polar Lipids, Alabaster, Alabama, USA.

Any anionic macromolecule can be transfected into a cell using a compound of formula I. An anionic macromolecule is a macromolecule which contains at least one negative charge per molecule. Examples of anionic macromolecules which can be transfected in accordance with this invention include polynucleotides, such as

5 deoxyribonucleic acids (DNA) and ribonucleic acids (RNA); and proteins, such as ribonucleoproteins and proteins used for immunization, e.g. viral proteins. Examples of DNA for use in the present invention are plasmids and genes, especially those for which gene therapy protocols have been launched such as cystic fibrosis transmembrane regulator (CFTR), adenosine deaminase (ADA), thymidine kinase (tk) and HLA B7; as well as

10 reporter genes such as beta-galactosidase, luciferase, green fluorescence protein (gfp), chloramphenicol transferase and alpha-1 antitrypsin. Other examples of DNA are oligodeoxynucleotides and their analogues used as antisense, aptamer or 'triple-helix' agents. Examples of RNA are ribozymes or oligoribonucleotide antisense molecules.

The nature of the cell which is to be transfected is not narrowly crucial. The cell can

15 be a procaryotic or eucaryotic cell, a mammalian or a plant cell.

In transfecting a cell using a conjugate of this invention, e.g. a compound of formula I, the cell is contacted with the anionic macromolecule in the presence of an appropriate amount of such compound. The appropriate amount of the conjugate, e.g. a compound of formula I for a given amount of anionic macromolecule depends on their respective

20 charges. The +/- charge ratio between the conjugate and the molecule to be transfected generally varies between 0.1-10, preferably between 0.5-5. The value of "+/- charge ratio" is calculated by dividing the number of positively charged groups on the amino acids in the group R³ by the number of negative charges of the molecule to be transfected. When the molecule to be transfected is a polynucleotide for example, number of negative charges

25 means the number of negatively charged phosphates in the backbone. The optimal ratio within these ranges depends on the cell to be transfected and is readily ascertained by one of skill in the art to which this invention pertains.

The amount of anionic macromolecules to the number of cells is such that the amount of anionic macromolecule for transfecting 10⁴ cells is from 0.1 ng to 10 mg,

30 preferably from 0.2 mg to 2 mg. When the anionic macromolecule is DNA the preferred amount of DNA for transfecting 10⁴ cells in vitro is from 0.1 mg to 10 mg. When cells are being transfected in vivo, the preferred amount of DNA is from 0.1 µg to 1 g.

In a preferred aspect of the invention the transfection is further carried out in the presence of a helper lipid and/or short chain phospholipid, and/or a cationic lipid or any

35 other known transfection competent molecule other than a conjugate of this invention.

Any conventional helper lipid can be used. Helper lipids are phospholipids which are known to increase delivery of macromolecules to cells when used together with known transfection competent molecules. Examples of helper lipids are phospholipids, such as phosphatidylcholines or phosphatidylethanolamines or mixtures thereof. Preferred helper lipids are phosphatidylethanolamines, such as dioleoylphosphatidylethanolamine. Any conventional short chain phospholipid can be used. Short chain phospholipids are phospholipids containing fatty acid residues, which fatty acid residues contain from 6 to 12 carbon atoms in their backbone. Examples of short chain phospholipids are phosphatidylcholines that carry two C₆₋₁₂ fatty acid residues. Preferred short chain phospholipids are dicapryl- and dicapryloyl phosphatidylcholine.

Examples of transfection competent molecules include cationic lipids as described by J.B. Behr in *Bioconjugate Chem.*, 1994, 5, 382-389 and X. Gao and L. Huang in *Gene Ther.*, 1995, 2, 710-722; polycations as described by A.V. Kabanov and V.A. Kabanov in *Bioconjugate Chem.*, 1995, 6, 7-20; peptides and polymers and other non-viral gene delivery systems as described by F.D. Ledley in *Human Gene Therapy*, 1995, 6, 1129-1144.

The helper lipid and/or short chain phospholipid and/or a cationic lipid or another known transfection competent molecule other than a conjugate of this invention is suitably in the form of a liposome, micelles, organic or aqueous dispersions, or organic or aqueous solutions. The optimal molar ratio between the compound of formula I and the helper lipid is 0.1-50, preferably 1-10. The optimal molar ratio between helper lipid and short-chain phospholipid is 2-20. The optimal molar ratio between the compound of formula I or II and additional transfection competent molecules is 0.1-10.

For transfection, an appropriate amount of a conjugate of this invention, e.g., a compound of formula I is added to the molecule to be transfected (e.g., plasmid DNA), suitably in an aqueous solution. Optionally, a helper lipid and, if desired, a short chain phospholipid and/or a cationic lipid or another known transfection competent molecule other than a conjugate of this invention is then added, either in form of liposomes, micelles, or as an organic solution or aqueous dispersion. Alternatively, the molecule to be transfected may be added to a composition comprising a compound in accordance with this invention, a helper lipid, and, if desired, a short chain phospholipid and/or a cationic lipid or another known transfection competent molecule other than a conjugate of this invention. The composition may be in solid, liquid, semisolid or aerosol form, suitably in form of liposomes, micelles, or as an organic solution or aqueous dispersion.

For transfecting cells in an animal or human patient the composition can be administered by oral, parenteral (i.v., i.m., s.c., i.d., i.p.) transdermal, pulmonary, nasal, rectal, ocular, ventricular, vascular (catheter) and intratumoral route. Furthermore, the composition can be administered by high velocity impactation administration to the skin surface. The progress of transfection can be measured by appropriate testing protocols which are known to those skilled in the art.

The present invention also refers to a composition comprising at least one compound as defined above and a helper lipid and/or a short chain phospholipid and/or a cationic lipid or another transfection competent molecule. In addition, the composition may comprise an anionic macromolecule, preferably a polynucleotide. These compositions may also comprise a polycationic polymer, preferably polyethylenimine. The components of these compositions may be in the form of an aqueous or organic solution, an aqueous or organic dispersion, or a liposome or a micelle.

The invention also relates to the use of a compound as above for transfecting a cell with an anionic macromolecule, preferably a polynucleotide.

In accordance with the present invention it has been found that conjugating a lipid to a basic, membrane disturbing peptide having a reversed amide bond results in novel compounds that bind polynucleotides and other anionic macromolecules and can be used for transfection of cells. Thus, the invention is concerned with a process for transfecting a cell with an anionic macromolecule containing, comprising contacting the cell with the anionic macromolecule in the presence of a compound as described above, so as to transfect the cell with the anionic macromolecule.

The following examples which are not limitative illustrate the invention further.

EXAMPLES

Example 1

a) Preparation of the retro-inverso peptide R³SH

Continuous-flow solid-phase synthesis was performed on a PioneerTM Peptide Synthesis System, starting from Tenta Gel S RAM resin (0.25 mmole/g [Rapp Polymere GmbH, Tübingen, Germany] according to the method described by Atherton and Sheppard, Solid Phase Peptide Synthesis : A Practical Approach (IRL Press Oxford 1989). The base-labile Fmoc group was used for α -amino protection. Side chains were protected with the following protection groups: D-Arg(Pbf), D-Cys(Trt), D-Gln(Trt), D-Lys(Boc), D-Ser(But), D-Thr(But) and D-Trp(Boc). Fmoc-amino acids (2.5 equiv.) were activated with an equivalent amount of TATU (L. A. Carpino J. Am. Chem. Soc. 1993, 115, 4397-4398) and DIPEA. Fmoc deprotection was achieved with 20% piperidine in DMF. D-Gln(Trt)-D-Gln(Trt)-D-Arg(Pbf)-D-Lys(Boc)-D-Arg(Pbf)-D-Lys(Boc)-D-Ile-D-Trp(Boc)-D-Ser(But)-D-Ile-D-Leu-D-Ala-D-Pro-D-Leu-Gly-D-Thr(But)-D-Thr(But)-D-Leu-D-Val-D-Lys(Boc)-D-Leu-D-Val-D-Ala-Gly-D-Ile-D-Cys(Trt)-amide Tenta Gel S-resin (2.0 g) was treated with a mixture (100 ml) of 90% TFA, 2% EDT, 5% H₂O, 3% triisopropylsilane for 6 hours. The reaction mixture was concentrated and poured into diethyl ether and the precipitate was collected by filtration and lyophilized from water. The crude peptide was purified by preparative RP-HPLC. There was obtained homogenous D-Gln-D-Gln-D-Arg-D-Lys-D-Arg-D-Lys-D-Ile-D-Trp-D-Ser-D-Ile-D-Leu-D-Ala-D-Pro-D-Leu-Gly-D-Thr-D-Thr-D-Leu-D-Val-D-Lys-D-Leu-D-Val-D-Ala-Gly-D-Ile-D-Cys-NH₂. 6 TFA.

b) Preparation of a compound of formula IV

The homogenous peptide obtained in paragraph a) above (24.2 mg, 7 mmole) was dissolved in a mixture of 2 ml of 100 mM ammonium acetate buffer, pH 6.5, and 2 ml of acetonitrile. To this solution there was added 7.4 mg of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[3-(2-pyridyldithio)propionate] in 0.5 ml of chloroform. The mixture was stirred at room temperature for 1 hour and the organic solvent was removed by evaporation. The remaining solution was washed 3 times with ethyl acetate and the aqueous phase was lyophilized. There was obtained 28.5 mg of the compound of formula IV wherein R¹ and R² are oleoyl and D-Gln-D-Gln-D-Arg-D-Lys-D-Arg-D-Lys-D-Ile-D-Trp-D-Ser-D-Ile-D-Leu-D-Ala-D-Pro-D-Leu-Gly-D-Thr-D-Thr-D-Leu-D-Val-D-Lys-D-Leu-D-Val-D-Ala-Gly-D-Ile-NH-CH[CONH₂](CH₂)- is R³. ISP-MS: M = 3722.

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Example 2

1 mg of the compound obtained in Example 1 was dissolved in 100 μ l acetonitrile and diluted in 900 μ l sterile pure water (1mg/ml). 15 μ l of a plasmid solution (1mg/ml) encoding the soluble human tumor necrosis factor receptor p55 gene was transferred into 1.5 ml medium and mixed. Various amounts of the compound obtained in a) was added, mixed and after 10 min at room temperature the mixture was transferred to 15 ml HEK293(EBNA) cells in suspension.

Table 1 shows the transfection efficiency of the compound as described in Example 1. The data clearly indicate that compound-mediated transfection can be achieved in the absence of detectable cell toxicity effects in serum-free suspension culture of 293(EBNA) cells.

Table 1

Transient tranfection of HEK293(EBNA) cells					
Compound (μ l/ml)	1	2	4	6	10
viability (%)	90 - 95	90 - 95	90 - 95	90 - 95	90 - 95
TNFRp55 (ng/ml)	0.1	0.2	2.5	6.3	83

Transient transfection of HEK293(EBNA) with various amounts of the compound of Example 1. Cells grown in serum-free suspension culture were transferred into fresh medium and transfected with an EBV-based vector encoding the soluble human tumor necrosis receptor p55 gene (TNFRp55). The released receptor protein was measured 72 hrs post-transfection in the medium and expressed as ng receptor per milliliter culture. The viability of the cells was determined by microscopic analysis.

Example 3

1 mg of the compound obtained in Example 1 was dissolved in 100 μ l acetonitrile and diluted in 900 sterile pure water (1mg/ml). 90 mg polyethelenimine (PEI), with a molecular weight of 25 kDa, were dissolved in sterile pure water (0.9 mg/ml), neutralized with HCl and sterile filtered and stored at room temperature.

3 μ l of a plasmid solution (1mg/ml) encoding the luciferase gene was diluted in 1.5 ml culture medium and mixed. Various amounts of the compound (1mg/ml) as described in Example 1 were added, mixed, followed by 8.3 μ l PEI (0.9 mg/ml). After mixing and incubation for 10 min at room temperature the mixture was transferred into 15 ml of various cells.

Table 2 shows the transfection efficiency of the compound described in Example 1 in combination with PEI by measuring the released receptor protein. The results clearly indicate the synergistic enhancement of the compound together with PEI.

Table 2

Transient transfection of HEK293(EBNA) cells together with PEI					
Compound + polyethylenimine (μ g/ml)	0.02 + 0.5	0.05 + 0.5	0.1 + 0.5	0.15 + 0.5	0 + 0.5
TNFRp55 (ng/ml)	490	800	1120	1100	180

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Transient transfection of HEK293(EBNA) with various amount of the compound of Example 1 in combination with polyethelenimine. Cells grown in serum-free suspension culture were transferred into fresh medium and transfected with an EBV-based vector encoding the soluble human tumor necrosis receptor p55 gene. The released receptor protein was measured 72 hours post-transfection in the medium and expressed as ng receptor per milliliter culture.

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Example 4

1 mg of the compound obtained in Example 1 was dissolved in 100 μ l acetonitrile and diluted in 900 μ l sterile pure water (1mg/ml). 90 mg polyethelenimine, with a molecular weight of 25 kDa, was dissolved in sterile pure water (0.9 mg/ml), neutralized with HCl and sterile filtered and stored at room temperature.

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3 μ l of a plasmid solution (1 mg/ml) encoding the luciferase gene was diluted in 1.5 ml culture medium and mixed. 2.25 μ l compound (1mg/ml) as described in Example 1 was

added, mixed, and followed by 8.3 µl PEI (0.9 mg/ml). After mixing and incubation for 10 min at room temperature the mixture was transferred into 15 ml of various cells.

Table 3 shows the transfection efficiency of the compound described in Example 1 in combination with PEI by measuring the luciferase activity. The results clearly indicate the synergistic enhancement of the compound together with PEI in various cell lines.

Table 3

Luciferase activity (RLU/ml)			
	Compound	Compound + PEI	PEI
BHK 21 C1	3.1×10^5	3.8×10^8	3.1×10^6
CHO dhfr-	7.1×10^5	1.8×10^8	1.1×10^7
HEK293 (EBNA)	2.2×10^6	6.1×10^9	9.9×10^7

Transient transfection of various commonly used cell lines with the compound of Example 1 in combination with polyethylenimine. Cells grown in serum-free suspension culture were transferred into fresh medium and transfected with a luciferase encoding vector. The luciferase activity was measured in the soluble cell extract after 24 hours incubation and expressed as RLU per milliliter culture.

Example 5

1 mg of the compound obtained in Example 1 was dissolved in 100 µl acetonitrile and diluted in 900 ul sterile pure water (1mg/ml). 90 mg polyethelenimine, with a molecular weight of 25 kDa, was dissolved in sterile pure water (0.9 mg/ml), neutralized with HCl and sterile filtered and stored at room temperature.

3 µl of a plasmid solution (1mg/ml) encoding the green fluorecence protein was diluted in 1.5 ml culture medium and mixed. 2.25 µl compound (1mg/ml) was added, mixed, and followed by 8.3 µl PEI (0.9 mg/ml). After mixing and incubation for 10 min at room temperature the mixture was transferred into 15 ml of various cells.

Table 4 shows the transfection efficiency of the compound described in Example 1 in combination with PEI by measuring the green fluorecence within the cells.

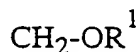
Table 4

Amount of fluorescence cells (%) - compound + PEI	
BHK 21 C1	25 (\pm 5)
CHO dhfr-	15 (\pm 5)
HEK293(EBNA)	85 (\pm 5)

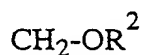
5 Transient transfection of various commonly used cell lines with the compound of Example 1 in combination with polyethylenimine. Cells grown in serum-free suspension culture were transferred into fresh medium and transfected with a green fluorescence protein encoding vector. The transfection efficiency was measured by counting the number of fluorescence cells after 24 hours incubation and expressed as percent transfected cells.

CLAIMS

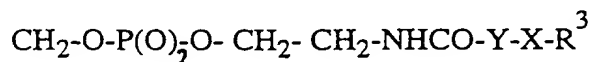
1. Conjugates of lipids and basic, membrane disturbing peptides which are compounds of formula



|



|



I

wherein R^1 and R^2 are a hydrocarbyl moiety of a straight-chain or branched-chain, saturated or unsaturated aliphatic carboxylic acid or a phospholipid moiety, R^3 is a basic, membrane disturbing peptide with a reversed amide backbone, Y is C_{2-10} alkylene, X is -C(O)-NH- or -S-S- , and salts thereof.

2. The compounds of claim 1 wherein R^1 and R^2 independently are an acyl moiety of a C_{12-20} carboxylic acid.

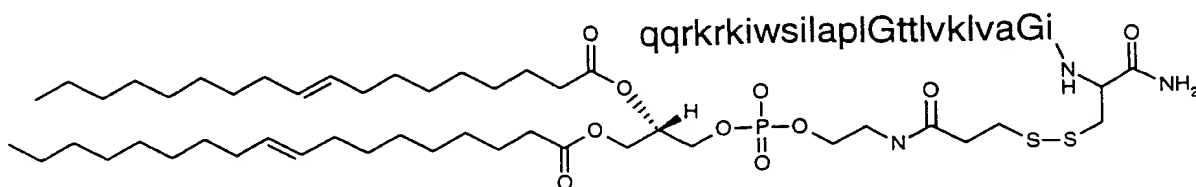
3. The compounds of claims 1 or 2 wherein R^1 and R^2 are independently selected from lauroyl, palmitoyl, stearoyl and oleoyl.

4. The compounds of claims 1 - 3 wherein X is -S-S- .

5. The compounds of claims 1 - 4 wherein R^3 is Gln-Gln-Arg-Lys-Arg-Lys-Ile-Trp-Ser-Ile-Leu-Ala-Pro-Leu-Gly-Thr-Thr-Leu-Val-Lys-Leu-Val-Ala-Gly-Ile-NH- $\text{CH[CONH}_2\text{]-(CH}_2\text{)-}$ with a reversed amide backbone or derivatives thereof consisting of at least 50 % D-amino acids.

6. The compounds of claim 1 - 5 wherein R^3 is D-Gln-D-Gln-D-Arg-D-Lys-D-Arg-D-Lys-D-Ile-D-Trp-D-Ser-D-Ile-D-Leu-D-Ala-D-Pro-D-Leu-Gly-D-Thr-D-Thr-D-Leu-D-Val-D-Lys-D-Leu-D-Val-D-Ala-Gly-D-Ile-NH- $\text{[CONH}_2\text{]-CH-(CH}_2\text{)-}$.

7. The compound according to any of claims 1 - 6, which is.



IV

9. The peptide Gln-Gln-Arg-Lys-Arg-Lys-Ile-Trp-Ser-Ile-Leu-Ala-Pro-Leu-Gly-Thr-Thr-Leu-Val-Lys-Leu-Val-Ala-Gly-Ile-Cys-NH₂ with a reversed amide backbone and consisting of at least 50 % D-amino acids or derivatives and/or salts and/or solvates thereof.

10. The peptide of claim 9 which is D-Gln-D-Gln-D-Arg-D-Lys-D-Arg-D-Lys-D-Ile-D-Trp-D-Ser-D-Ile-D-Leu-D-Ala-D-Pro-D-Leu-Gly-D-Thr-D-Thr-D-Leu-D-Val-D-Lys-D-Leu-D-Val-D-Ala-Gly-D-Ile-D-Cys-NH₂.

11. A composition comprising at least one compound as defined in any one of claims 1-8 and a helper lipid and/or a short chain phospholipid and/or a cationic lipid or another transfection competent molecule.

12. The composition of claim 11 comprising in addition an anionic macromolecule, preferably a polynucleotide.

13. The composition of claims 11 or 12 comprising in addition a polycationic polymer, preferably polyethylenimine.

14. The composition of claims 11 - 13, wherein the components are in the form of an aqueous or organic solution, an aqueous or organic dispersion, or a liposome or a micelle.

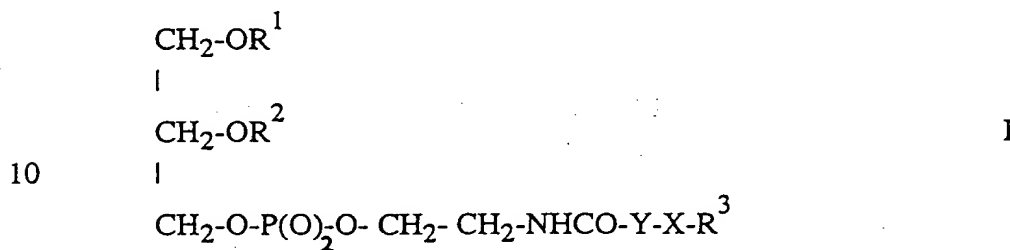
15. Use of a compound as defined in any one of claims 1-8 for transfecting a cell with an anionic macromolecule, preferably a polynucleotide.

16. A process for transfecting a cell with an anionic macromolecule, comprising contacting a cell with the anionic macromolecule in the presence of a compound as defined in any of claims 1 - 8.

Ref. 20'083

Abstract

The invention relates to conjugates of lipids and basic, membrane disturbing peptides, characterized in that the peptides comprise a reversed amide backbone, particularly compounds of the formula



wherein R¹ and R² are a hydrocarbyl moiety of a straight-chain or branched-chain, saturated or unsaturated aliphatic carboxylic acid or a phospholipid moiety, R³ is a basic, membrane disturbing peptide with a reversed amide backbone, Y is C₂₋₁₀ alkylene, X is -C(O)-NH- or -S-S-, and salts thereof.

